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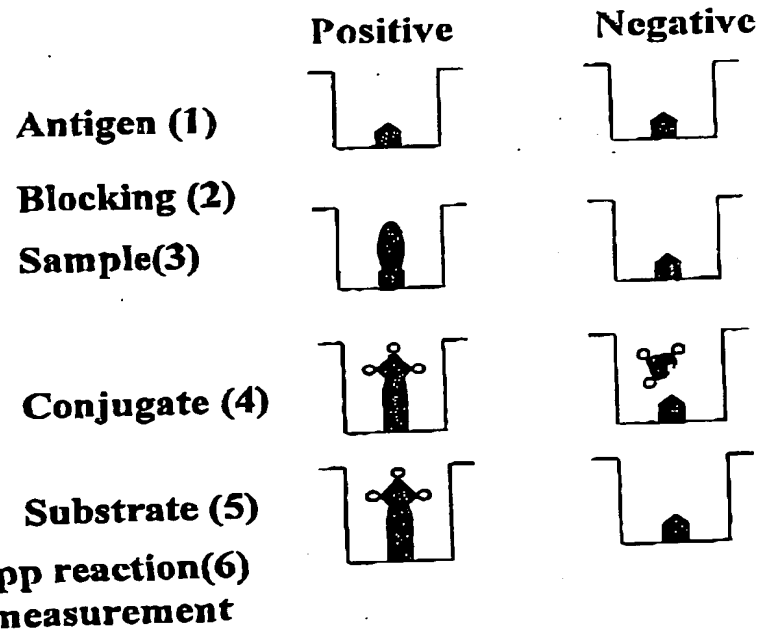
(57) Abstract

The present invention relates to a method and kit of detecting antibodies in the serum of animals infected with equine infectious anemia virus using the immunodiagnosis with the recombinant viral antigen gp90. The antigen was bound to solid supports (microtitter plates, tubes, beads or nitrocellulose papers or nylon) and reacted with clinical samples. After incubation with conjugated (Anti-equine immunoglobulin-enzyme) the reaction was revealed with a solution composed of the substract of the enzyme used in the conjugate (cromogene). After development of the reaction (color formation) it was stopped with acid solution and measured. The immunoassay may be a direct second antibody immunoassay, and one or two step sandwich immunoassay.



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THE IMMUNOENZYMATIC ASSAY FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING RECOMBINANT PROTEIN (rGP90) DERIVED FROM EQUINE INFECTIOUS ANEMIA VIRUS.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method of detecting antibodies against envelope surface antigen of equine infectious anemia virus (EIAV), using as antigen the non glycosylated recombinant protein (rgp90) in immunoenzymatic assays. More particularly, it relates to the use of recombinant protein gp 90 in kits for diagnosis of equine infectious anemia (EIA).

BACKGROUND OF THE INVENTION

The equine infectious anemia (EIA) is one of the oldest diseases caused by virus, having been described for the first time in France by LIGNEE (**Rec. Med. Vet.**, 20:30, 1843) and recognized as viral disease by VALLEE and CARRE (**Acad. Sci.** ,139:331-333,1904). The disease affects exclusively the members of the family **Equidae** presenting a worldwide distribution and of great economical importance consequently .

The EIA virus (EIAV) is classified as a lentivirus of the **Retroviridae** family (CHARMAN et al. **J. Virol.** 19(2):1073 -1076,1976), it is genetic and antigenically related to the other lentiviruses which are characterized by developing persistent infection in host. The EIA has played a specially important role in comparative virology and in the studies of the acquired immunodeficiency syndrome (AIDS). Besides their morphological identity, both viruses are similar in terms of nucleotide sequences that code for structural surface proteins. These group of virus present genetic and antigenic variants during persistent infections, which is associated to immune response scape (MONTAGNIER et al. **Ann. Virol.**, 135:119-134, 1984, MONTELARO et al. **J. Biol. Chem.**, 259:10539-10544,1984, RUSHLOW et al. **Virology**, 155:309-321, 1986, STREICHER et al. **J. Am. Med. Assoc.** 256:2390-2391, 1986, STOLER et al. **J. Am. Med. Assoc.** 256:2360-2364,1986 and HAHN et al. **Science**, 232:1548-1553, 1986).

The transmission of EIAV occurs mainly through bite of arthropods vectors (tabanideos) which inoculate the virus into the animal's blood stream (mechanical transmission) when feeding themselves. The way of transmition is

responsible for the high prevalence of EIA in areas favorable to the life cycle of vectors (ISSEL et al. **Vet..** 17:251-286, 1988). The EIAV can also be transmitted by the placenta and colostro of mares with high virus levels, and by needles and surgical instruments contaminated with blood (COGGINS **Comparative diagnosis of viral diseases,NY**, 4:646-658, 1981). The course of infections shows different clinical forms of the disease (subacute, chronic and mainly inaparent or assymptomatic) in horses (ISSEL & COGGINS, **J. Am. Vet. Med. Assoc.** 174(7):727-33, 1979) and the most prominent signs are the fever episodes, hemolytic anemia, anorexia, fast weight loss and ventral edema.

The laboratory diagnosis plays a decisive role in the control and prevention of EIA if considering the high prevalence of assymptomatic carriers, non conclusive and possibility to confuse clinical diagnosis with other diseases as the trypanosomiasis, pyroplasmosis, leptospirosis, hepatitis and by parasites .

The diagnose of EIAV has been done through the detection of specific antibodies against surface antigen of virus present in the serum of affected animals by using the Coggins or agar gel diffusion test (U.S.Pat. nro.3,929,982 and U.S.Pat. No. 3,932,601). In the Coggins test the antigen and sample serum is placed side by side in an agarose gel plate. If EIA antibodies are present in the test serum, they will form a precipitin line when diffusing toward the agarose gel .

This methodology is inherently insensitive since EIAV antigen preparation derived from spleen of infected animals or equine derme cultures cells may be contaminated with non-EIAV antigens during its preparation. Besides, antibodies against non-EIAV antigens may be present in the test serum and can react with the non-EIA antigens forming a variety of nonspecific precipitin lines. Even if, EIAV-antigen batches can be purified the Coggins test is laborious, time-consuming and demanding of considerable expertise in interpretation of results. The Coggins test procedure takes twenty-four to forty-eight hours for the formation of clearly visible precipiting lines, delayinf results.

Porter (U.S.Pat.No.4,806,467) discloses a method for detecting the EIA virus using a competitite enzyme-linked immunoabsorbent assay incorporating a purified viral antigen and a monoclonal antibody. To obtain the antigen, the EIAV must first be cultured. The antigen used was p26 core protein of the EIAV

and is obtained through (purification of the cultured virus by a variety of means) well known in the art. The technique of virus tissue cultures increases the possibility of assay yield false positive results since the virus may be contaminated with other forms of protein or even another virus. Additionally, the EIAV is hard to culture, making Porter's approach very difficult for large scale production.

The use of a synthetic peptide in an enzyme linked immunosorbent assay for the detection of human immunodeficiency virus (HIV) was disclosed by Shoeman, R.L. et al (Analytical Biochemistry 161:370-379,1987).

Darrel & Peisheng, the U.S. Patent No. 5,427,907, discloses a method to use a synthetic peptide as the antigen in an immunoassay for the detection of antibodies against the equine infectious anemia virus in the serum of horses. This procedure include only the search of some epitopes of a virus proteins.

It is an object of the present invention to provide an assay for the detection of the equine infectious anemia virus antibodies which may be fast, easily and quickly performed by using the stable recombinant envelope protein (rgp90) which may be produced in sufficient amounts at a low cost.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows schematically the method of diagnosis

Figure 2 shows the titration of positive and negative sera in Elisa with the recombinant protein rgp90 as antigen.

Figure 3 demonstrates the distribution of the optical density (OD) in Elisa with the recombinant protein gp90 as antigen with 84 positive and 70 negative horses samples, previously tesyed by IDGA and ELISA by using EIAV-antigen produced in cell cultures

Figure 4 demonstrates the optical density of the ELISA reaction with the recombinant protein gp90 as antigen after EIAV "Wyoming" strain experimental infected-horse.

DETAILED DESCRIPTION OF THE INVENTION

It is, therefore , an object of the present invention to provide a method of immunodiagnosis for EIA disease that uses the recombinant protein gp90 corresponding derived from viral envelope of EIAV. The method consists of binding the recombinant antigen to solid supports (microtiter plates, tubes, beads or nitrocelullose or nylon papers or any kind that allow protein binding) and to proceed the analysis of the sera (presence of antibodies) from animals suspected of infection with the EIAV.

The recombinant protein gp90 is added to a solid phase support and incubated for sufficient time to ensure that protein was bound to the support. The equine test sample is added to the support and incubated for a period of time sufficient to permit that any EIA-antibodies are removed from sample.

Labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. Labeled conjugate is added wich binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. High level of bound conjugate indicates a positive result, which mean presence of EIA viral antibodies. A low level of bound conjugate indicates a negative result which mean ausence or undetectable level of EIA viral antibodies..

A variety of commercially available solid phase supports may be used for protein binding. The direct binding of equine antibodies present in the test serum to the solid phase support is likely to result in a false positive reading. To prevent such binding, the blocking solution is used to fill any empty binding sites on the support which did not bind antibodie-protein. Any substance which will not react with EIA viral antibodies and antigen will function as a blocker. A conjugate is something which will recognize and bind with the test serum EIA viral antibody.

The conjugate may be labeled using a variety of labeling means, including but not limited to: enzyme labeling, fluorescent labeling, and magnetic labeling. If enzymatic labeling is the labeling means chosen, the conjugate is labeled with an enzyme preferably select from the group consisting of horseradish peroxidase and alkaline phosphatase. Other enzymes may be used.

When an enzyme label is used, the labeled conjugate is detected by adding an amount of a substrate which will recognize and react with the enzyme label to form a product that will produce a color change visible to the naked eye. The presence of color indicates a sufficient level of test serum antibodies to indicate infection. An absence of color is an indicator of a lack of infection, as the animal did not produce a significant number of antibodies to the virus. Hence, the labeled conjugate had few antibodies, if any, to bind with and was subsequently removed from the support. There are a variety of both peroxidase and phosphatase substrates which will react with horseradish peroxidase and alkalinie phosphatase enzymes, respectively to form a colored product.

A preferred peroxidase substrate is an ortho-phenylenediamine/hydrogen peroxide solution. The intensity of the color of the product may be quantified using a spectrophotometer to read absorvance. Howewer, measuring the absorvance is not necessary to obtain an accurate reading of the results of the assay.

The titration of positive and negative sera in Elisa with 1µg recombinant protein gp90 as antigen (Figure 2) shows the detection of antibodies anti-gp90 in the ELISA test using dilutions of the serum from 1:4 to 1:256 and obtaining OD from 0.800 to 0.400. The negative controls demonstrate that there is a non specific reaction.

The optical density obtained when sera from 84 positive and 70 negative horses were tested is presented on Figure 3, showing the frequency of the different optical densities obtained.

An animal was experimentally infected and its sera tested with the ELISA rgp90. Figure 4 shows that specific antibodies were detected seven days after the infection together with the appereance of fever.

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples illustrate the present invention and are not intended to limit it in spirit or scope.

EXAMPLE 1

The process can be better understood through the following description in consonance with the illustration in Figure1 where the binding of the antigen (recombinant protein gp90) to the solid support (1), it is done by its dilution in

carbonate buffer (Na_2CO3 0.1-0.5M; NaHCO3 0.1-0.5M, pH 8.0-9.6), added in the concentrations of 0.01-1g and incubated the temperature of 4-8°C for 18-24 hours in micro-technique plates, tubes or beads followed by electrotransference or passive transference to nitrocelullose or nylon supports. After antigen binding, the support was washed for 3 to 6 times with buffer solution (0.01-0.02 M NaH_2PO4 , 0.01-0.02 MNa_2HPO4 , 0.02-0.04M KCl, 0.85-0.9% NaCl pH 7.0-7.5),and then with 0.05-0.1% of tween-20 (Buffer-Tween). To block the inespecific sites of binding (2) the used support was incubated with block solution (skimmed powdered milk 1-5% bovine , 1-5%albumin or 1-5% casein in Tween buffer) for 30-60 min at 23°C-37°C. After a new wash of the support with Tween buffer, as described previously, the positive and negative control and the serum samples were diluted in Tween buffer, to bound to the antigen linked to the solid support (3), and incubated at 23°C-37°C. After new wash of the support with Tween buffer, the conjugate was added, where the anti-equine immunoglobuline binds to the antibodies that are tied up to the antigens (4). Conjugate can be an equine anti-immunoglobuline conjugated to the enzyme peroxidase or any other enzyme as acetylcolinesterase, lactate desidrogenase, galactosidase, glicose oxidase, alkaline fosfatase, or another. This conjugate was diluted in Tween buffer in agreement with its title and added to the support and then incubated at23°C-37°C for 30-60 min. A new wash of the support with Tween buffer and the development of the reaction was proceeded (5) with the enzyme of the conjugate, transforms the substrate of colorless to a red-faced product. The developing solution is composed of the substrate of the enzyme used in the conjugate that for the peroxidase for example is the ortofenilenodiamino diluted in phosphate or citrate buffer 0.1-0.2 M, pH 5.0-8.0. After the color development, which is proportional to the concentration of specific antibodies in each sample, solution of acid was used (sulfuric acid) for stop-reaction (6), where the acid interrupts the previous reaction. For the final result the measurement (7) of the color intensity formed in each reaction (sample) was made. This reading was made visually or in espectrophotometer, in absorbance, with a specific filter for the color formed by the developing solution.

EXAMPLE 2

The kit for diagnosis of the EIAV may contain the the folowing products: (a) the antigen recombinant gp90 from EIA coated to the solid support (microplate, microtiter wells, tubes, capillary tubes, sticks, dipsticks, beads) with different chemical composition (polystyrene, polypropilene, polyethylene, polypropylene, poly-carbonate, polyvinyl, polystyrene, latex, nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass (b) the anti-equine immunoglobulin conjugated with label that is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin (c) the substrate for the label as orthophenilenodiamine and H_2O_2 (d) a blocking solution (0.01-0.02M NaH_2PO4, 0.01-0.02M Na_2HPO4 , 0.02-0.04M KCl, 0,85-0,9% NaCl pH 7.0-7.5), with 0.05-0.1% of Tween 20 and skimmed powdered milk 1-5% bovine , 1-5% albumin or 1-5%casein (e) a diluent solution for specimen and conjugate (0.01-0.02 M NaH_2PO4 , 0.01-0.02M Na_2HPO4 , 0.02-0.04M KCl , 0.85-0.9% NaCl pH 7.0-7.5), with 0.05-0.1% of Tween 20 and 1% skimmed powdered milk (f) a diluent solution for substrate 0.1M Na_2HPO4 , 0.1M C6H8O7 pH 5,0 (f) stop solution 7N H_2SO4 (g) wash solution (0.01-0.02M NaH_2PO4, 0.01-0.02M Na_2HPO4, 0.02-0.04 M KCl , 0.85-0.9% NaCl pH 7.0-7.5), with 0.05-0,1% of Tween 20 (h) positive control inactivated horse serum (I) negative control inactivated horse serum

While the present invention has been described in connection with an example, it will be understood that modifications and variations apparent to those ordinary skill in the art are within the scope of the present invention.

WHAT IS CLAIMED IS:

1. An immunoenzymatic assay for detection of antibody by using the equine infectious anemia virus recombinant gp90 envelope antigen in animal test samples comprising:

(a) binding of the recombinant gp90 envelope antigen to a solid support,

(b) reacting the bound antigen with a test sample of serum,

(c) removing the unbound test sample,

(d) reacting the bound test antibody with a labeled antibody

(e) measuring the amount of bound antibody specific to the equine anemia infectious virus gp 90 envelope antigen in the test sample

2. The immunoassay according to claim a, wherein said label is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin.

3. The immunoassay according to claim 1, wherein said solid support is selected from the group consisting of polystyrene or polypropilene microtiter wells, polyethylene, polypropylene, polycarbonate, polyvinyl, polystyrene, or glass test tubes, capillary tubes, dipsticks, or beads; latex beads; nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 97/00083

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: G 01 N 33/543, 33/541, 33/535, 33/533, 33/566, 33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Vol.124, No.13, 25 March 1996 (Columbus, Ohio, USA), page 993, column 1, abstract No.172919b, GRUND, C.H. et al.:"Fine specificity of equine infectious anemia virus gp90-specific antibodies associated with protective and enhancing immune responses in experimentally infected and immunized ponies", & J.Gen.Virol. 1996, 77(3), 435-42 (Eng).	1,2
A	Chemical Abstracts, Vol.116, No.11, 16 March 1992 (Columbus, Ohio, USA), page 559, column 2, abstract No.103966n, BALL, J.M. et al.:"Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infections anemia virus by using synthetic peptide strategies", & J.Virol. 1992, 66(2), 732-42 (Eng).	1,2
A	Chemical Abstracts, Vol.108, No.19, 09 May 1988 (Columbus, Ohio, USA), page 503, column 1, abstract No.165830g, O'ROURKE, K. et al.:"Antiviral, anti-glycoprotein and neutralizing antibodies in foals with equine infectious anemia virus", & J.Gen.Virol. 1988, 69(3), 667-74 (Eng).	1,2

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 932 601 A (COGGINS) 13 January 1976 (13.01.76), totality.	1

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/BR 97/00083

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
US A 3932601	13-01-76	CA A1 1020458 US A 3929982 AU A1 40838/72	08-11-77 30-12-75 11-10-73

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well known in the art. The technique of virus tissue cultures increases the possibility of assay yield false positive results since the virus may be contaminated with other forms of protein or even another virus. Additionally, the EIAV is hard to culture, making Porter's approach very difficult for large scale production.

The use of a synthetic peptide in an enzyme linked immunosorbent assay for the detection of human immunodeficiency virus (HIV) was disclosed by Shoeman, R.L. et al (Analytical Biochemistry 161:370-379,1987).

Darrel & Peisheng, the U.S. Patent No. 5,427,907, discloses a method to use a synthetic peptide as the antigen in an immunoassay for the detection of antibodies against the equine infectious anemia virus in the serum of horses. This procedure include only the search of some epitopes of a virus proteins.

It is an object of the present invention to provide an assay for the detection of the equine infectious anemia virus antibodies which may be fast, easily and quickly performed by using the stable recombinant envelope protein (rgp90) which may be produced in sufficient amounts at a low cost.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows schematically the method of diagnosis

Figure 2 shows the titration of positive and negative sera in Elisa with the recombinant protein gp90 as antigen.

Figure 3 demonstrates the distribution of the optical density (OD) in Elisa with the recombinant protein gp90 as antigen with 84 positive and 70 negative horse serum samples, previously tested by IDGA and ELISA by using EIAV-antigen produced in cell cultures

Figure 4 demonstrates the optical density of the ELISA reaction with the recombinant protein gp90 as antigen after EIAV "Wyoming" strain experimental infected-horse.

DETAILED DESCRIPTION OF THE INVENTION

It is, therefore , an object of the present invention to provide a method of immunodiagnosis for EIA disease that uses the recombinant protein gp90 corresponding derived from viral envelope of EIAV. The method consists of binding the recombinant antigen to solid supports (microtiter plates, tubes, beads or nitrocelullose or nylon papers or any kind that allow protein binding) and to proceed the analysis of the sera (presence of antibodies) from animals suspected of infection with the EIAV.

The recombinant protein gp90 is added to a solid phase support and incubated for sufficient time to ensure that protein was bound to the support. The equine test sample is added to the support and incubated for a period of time sufficient to permit that any EIA-antibodies are removed from sample.

Labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. Labeled conjugate is added wich binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. High level of bound conjugate indicates a positive result, which mean presence of EIA viral antibodies. A low level of bound conjugate indicates a negative result which mean ausence or undetectable level of EIA viral antibodies..

A variety of commercially available solid phase supports may be used for protein binding. The direct binding of equine antibodies present in the test serum to the solid phase support is likely to result in a false positive reading. To prevent such binding, the blocking solution is used to fill any empty binding sites on the support which did not bind antibodie-protein. Any substance which will not react with EIA viral antibodies and antigen will function as a blocker. A conjugate is something which will recognize and bind with the test serum EIA viral antibody.

The conjugate may be labeled using a variety of labeling means, including but not limited to: enzyme labeling, fluorescent labeling, and magnetic labeling. If enzymatic labeling is the labeling means chosen, the conjugate is labeled with an enzyme preferably select from the group consisting of horseradish peroxidase and alkaline phosphatase. Other enzymes may be used.

When an enzyme label is used, the labeled conjugate is detected by

adding an amount of a substrate which will recognize and react with the enzyme label to form a product that will produce a color change visible to the naked eye. The presence of color indicates a sufficient level of test serum antibodies to indicate infection. An absence of color is an indicator of a lack of infection, as the animal did not produce a significant number of antibodies to the virus. Hence, the labeled conjugate had few antibodies, if any, to bind with and was subsequently removed from the support. There are a variety of both peroxidase and phosphatase substrates which will react with horseradish peroxidase and alkalinie phosphatase enzymes, respectively to form a colored product.

A preferred peroxidase substrate is an ortho-phenylenediamine/hydrogen peroxide solution. The intensity of the color of the product may be quantified using a spectrophotometer to read absorbance. However, measuring the absorbance is not necessary to obtain an accurate reading of the results of the assay.

The titration of positive and negative sera in Elisa with 1µg recombinant protein gp90 as antigen (Figure 2) shows the detection of antibodies anti-gp90 in the ELISA test using dilutions of the serum from 1:4 to 1:256 and obtaining OD from 0.800 to 0.400. The negative controls demonstrate that there is a non specific reaction.

The optical density obtained when sera from 84 positive and 70 negative horses were tested is presented on Figure 3, showing the frequency of the different optical densities obtained.

An animal was experimentally infected and its sera tested with the ELISA rgp90. Figure 4 shows that specific antibodies were detected seven days after the infection together with the appearance of fever.

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples illustrate the present invention and are not intended to limit it in spirit or scope.

EXAMPLE 1

The process can be better understood through the following description in consonance with the illustration in Figure1 where the binding of the antigen (recombinant protein gp90) to the solid support (1), it is done by its dilution in carbonate buffer (Na_2CO3 0.1-0.5M; NaHCO3 0.1-0.5M, pH 8.0-9.6), added in

the concentrations of 0.01-1µg and incubated the temperature of 4-8°C for 18-24 hours in micro-technique plates, tubes or beads followed by electrotransference or passive transference to nitrocelullose or nylon supports. After antigen binding, the support was washed for 3 to 6 times with buffer solution (0.01-0.02 M NaH_2PO4 , 0.01-0.02 MNa_2HPO4 , 0.02-0.04M KCl, 0.85-0.9% NaCl pH 7.0-7.5),and then with 0.05-0.1% of tween-20 (Buffer-Tween). To block the inespecific sites of binding (2) the used support was incubated with block solution (skimmed powdered milk 1-5% bovine , 1-5%albumin or 1-5% caseín in Tween buffer) for 30-60 min at 23°C-37°C. After a new wash of the support with Tween buffer, as described previously, the positive and negative control and the serum samples were diluted in Tween buffer, to bound to the antigen linked to the solid support (3), and incubated at 23°C-37°C. After new wash of the support with Tween buffer, the conjugate was added, where the anti-equine immunoglobuline binds to the antibodies that are tied up to the antigens (4). Conjugate can be an equine anti-immunoglobuline conjugated to the enzyme peroxidase or any other enzyme as acetylcolinesterase, lactate desidrogenase, galactosidase, glicose oxidase, alkaline fosfatase, or another. This conjugate was diluted in Tween buffer in agreement with its title and added to the support and then incubated at23°C-37°C for 30-60 min. A new wash of the support with Tween buffer and the development of the reaction was proceeded (5) with the enzyme of the conjugate, transforms the substrate of colorless to a red-faced product. The developing solution is composed of the substrate of the enzyme used in the conjugate that for the peroxidase for example is the ortofenilenodiamino diluted in phosphate or citrate buffer 0.1-0.2 M, pH 5.0-8.0. After the color development, which is proportional to the concentration of specific antibodies in each sample, solution of acid was used (sulfuric acid) for stop-reaction (6), where the acid interrupts the previous reaction. For the end result the measurement (7) of the color intensity formed in each reaction (sample) was made. This reading was made visually or in espectrophotometer, in absorbance, with a specific filter for the color formed by the developing solution.

EXAMPLE 2

The kit for diagnosis of the EIAV may contain the the folowing products: (a) the antigen recombinant gp90 from EIA coated to the solid support (microplate,

microtiter wells, tubes, capillary tubes, sticks, dipsticks, beads) with different chemical composition (polystyrene, polypropilene, polyethylene, polypropylene, poly-carbonate, polyvinyl, polystyrene, latex, nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass (b) the anti-equine immunoglobulin conjugated with label that is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin (c) the substrate for the label as orthophenilenodiamine and H_2O_2 (d) a blocking solution (0.01-0.02M NaH_2PO4, 0.01-0.02M Na_2HPO4 , 0.02-0.04M KCl, 0,85-0,9% NaCl pH 7.0-7.5), with 0.05-0.1% of Tween 20 and skimmed powdered milk 1-5% bovine , 1-5% albumin or 1-5%casein (e) a diluent solution for specimen and conjugate (0.01-0.02 M NaH_2PO4 , 0.01-0.02M Na_2HPO4 , 0.02-0.04M KCl , 0.85-0.9% NaCl pH 7.0-7.5), with 0.05-0.1% of Tween 20 and 1% skimmed powdered milk (f) a diluent solution for substrate 0.1M Na_2HPO4 , 0.1M C6H8O7 pH 5,0 (f) stop solution 7N H_2SO4 (g) wash solution (0.01-0.02M NaH_2PO4, 0.01-0.02M Na_2HPO4, 0.02-0.04 M KCl , 0.85-0.9% NaCl pH 7.0-7.5), with 0.05-0,1% of Tween 20 (h) positive control inactivated horse serum (I) negative control inactivated horse serum

While the present invention has been described in connection with an example, it will be understood that modifications and variations apparent to those ordinary skill in the art are within the scope of the present invention.

AMENEDED SHEET

WHAT IS CLAIMED IS:

1. An immunoenzymatic assay for detection of antibody by using the equine infectious anemia virus recombinant gp90 envelope antigen in animal test samples comprising:

(a) the use of the recombinant gp90 (rgp90) envelope protein from the equine infectious anemia virus,

(b) binding of the recombinant gp90 envelope antigen to a solid support,

(c) reacting the bound antigen with a test sample of serum,

(d) removing the unbound test sample,

(e) reacting the bound test antibody with a labeled antibody

(f) measuring the amount of bound antibody specific to the equine anemia infectious virus gp 90 envelope antigen in the test sample

2. The immunoassay according to claim a, wherein said label is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin.

3. The immunoassay according to claim 1, wherein said solid support is selected from the group consisting of polystyrene or polypropilene microtiter wells, polyethylene, polypropylene, polycarbonate, polyvinyl, polystyrene, or glass test tubes, capillary tubes, dipsticks, or beads; latex beads; nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference ÅL/Ru 40250	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/BR97/00083	International filing date *(day/month/year)* 19.12.1997	Priority date *(day/month/year)* 18.12.1996
International Patent Classification (IPC) or national classification and IPC6 G 01 N 33/543, G 01 N 33/569		
Applicant Universidade Federal de Minas Gerais et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20.07.1998	08.04.1999
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Telex 17978 PATOREG-S Facsimile No. 08-667 72 88	Authorized officer Carl-Olof Gustafsson Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/BR97/00083

I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

☐ the international application as originally filed.

☒ the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages 1-7, filed with the letter of 15.03.1999.
pages _____, filed with the letter of _____.

☒ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-3, filed with the letter of 15.03.1999.
Nos. _____, filed with the letter of _____.

☒ the drawings, ~~sheets~~/fig 1-4, as originally filed,
~~sheets~~/fig _____, filed with the demand
~~sheets~~/fig _____, filed with the letter of _____.
~~sheets~~/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, ~~sheets~~/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/BR97/00083

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-3	YES
	Claims		NO
Inventive step (IS)	Claims	1-3	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-3	YES
	Claims		NO

2. Citations and explanations

The present application refers to an immunoenzymatic assay for detection of antibodies to equine infectious anaemia virus (EIAV) in a sample with the aid of a recombinant gp90 (rgp90) envelope solid phase bound antigen.

The International Search Report revealed three abstracts and a patent document relating to the general state of the art. The corresponding articles are referred to below.

Grund CH et al. in J. Gen. Virol. vol. 77, no 3, 1996, pp 435-42 have used a "standard gp90 peptide ELISA" test to detect EIAV antibodies in samples from infected ponies by means of a "standard gp90 peptide ELISA". The standard ELISA assay involves synthetic parts of the gp90 molecule (see fig. 1) bound to a solid support. Bound antibodies are indicated with the aid of a labelled secondary antibody. Grund et al. state that their rationale for evaluating peptide specificites is "the complex array of conserved and variable linear antigenic determinants recognised" (p 437 left col.).

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.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

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US3932601, involves the use of gel-immunodifusion assays for EIAV antibodies.

However, a second patent document, US5427907, see col. 1, ll 62-66 and example 1, mentioned in the present description, is considered to be more relevant in the context of the present application. This document reveals an assay for EIAV antibodies with the aid of solid phase bound synthetic gp45 peptide. In col 11, ll 1-7, it is stated that gp90 would not be an adequate antigen in this context as it is "known to exhibit antigenic variation".

The claimed immunoassay is novel and industrially applicable. It differs from the known assays in that the entire rgp90 molecule is used as solid phase bound antigen instead of synthetic peptide fragments of the gp90 molecule or synthetic gp45 peptide fragments.

Providing a fast and easy assay for EIAV antibodies in horses is considered to be the problem solved by the method of the present application. As revealed by the above cited documents this problem has been solved by using *synthetic gp45/gp90 peptide fragments* as solid phase bound reagent. It may seem obvious to a person skilled in the art to use the recombinantly produced gp90 mentioned by Grund. et al. or Ball et al.. However, in view of the drawbacks of gp90 referred to in US5427907 and the unexpectedly early detectable response achieved with the present method as compared to the known methods, an inventive step is considered to be present.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 August 1998 (12.08.98)	
International application No. PCT/BR97/00083	**Applicant's or agent's file reference**
International filing date (day/month/year) 19 December 1997 (19.12.97)	**Priority date** (day/month/year) 18 December 1996 (18.12.96)
Applicant PEREGRINO FERREIRA, Paulo, César et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 July 1998 (20.07.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Ingrid Hours

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

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(PCT Article 36 and Rule 70)

Applicant's or agent's file reference AL/Ru 40250	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
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PCT/BR97/00083

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